APPLICATION OF THE LUCIFERIN-LUCIFERASE ENZYME SYSTEM FOR DETERMINATION OF ADENOSINE TRIPHOSPHATE (ATP) TO STUDIES ON THE MECHANISMS OF HERBICIDE ACTION

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Adenosine triphosphate (ATP) pivotally supplies energy for biosynthetic reactions. Photosynthetic and oxidative phosphorylation are the two major processes through which a chlorophyllous organism produces ATP. Despite many reports of herbicidal effects on these processes in vitro, much less is known about herbicidal effects on phosphorylation processes in vivo. We have used the firefly luciferin-luciferase assay, which measures ATP directly, to study herbicide effects on phosphorylation processes in vivo. A detailed study of our method, as well as the use of the Aminco Chem-Glow Photometer,* has been published elsewhere (St. John, 1970). Briefly, our system is as follows:

1. ATP is extracted by boiling for 1 minute in a suitable solvent. We have found absolute ethanol or water or a combination of the two to be most suitable for algae and other plant materials.
2. The extracts are blown dry with nitrogen and reconstituted with water.
3. A 1-ml fraction is diluted to 2 ml and made 0.025 M in HEPES-Mg^{++} buffer, pH 7.5.
4. A second 1-ml fraction is treated similarly, but a known amount of ATP is added.
5. By use of an Aminco Chem-Glow Photometer attached to an integrator-timer, light emission is integrated for 30 seconds after the mixing of a 0.45-ml sample with 0.1-ml reconstituted, commercially available, firefly lantern extract.

* Mention of a trade name or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

6. ATP concentration is determined by solution of this equation:

$$C\mu = \frac{I\mu\, C_s\, D_f}{I\mu + C - I\mu} \qquad (1)$$

Here $C\mu$ is the ATP concentration in moles per liter, $I\mu$ is the relative integrated intensity produced in response to the unknown concentration of ATP, C_s is the known concentration of ATP in moles per liter, D_f is a dilution factor, and $I\mu + C$ is the relative integrated intensity produced in response to the unknown plus the known concentration of ATP.

This method has several advantages. The method of constant addition for quantitation of ATP successfully overcomes interference caused by substances normally found in biological extracts, the added herbicides, the solvent used for extraction, and such added things as ATP, AMP, GTP, and PP. Thirty-second integration of the light emitted after the mixing of the sample and enzyme, which includes the peak intensity of the initial flash and a portion of the decaying light,

- Minimizes the variables of injection and sample mixing;
- Is highly reproducible, with less than a 2 percent relative standard deviation;
- Affords a level of sensitivity equal to that obtained with purified luciferin-luciferase preparations—namely, 10^{-9} M ATP solutions or picomole quantities of ATP with linearity extending over at least a thousand-fold range; and
- Costs about 3 cents a test as compared with 30 cents a test when purified luciferin-luciferase preparations are required.

To evaluate herbicide effects on phosphorylation processes in vivo, we selected *Chlorella* as the test organism and diuron [3 - (3,4-dichlorophenyl) -1, 1-dimethylurea] and chlorpropham (isopropyl *m*-chlorocarbanilate) as test chemicals (St. John, 1971). When *Chlorella* cells were grown in the light on a totally inorganic medium, conditions that favor photosynthetic phosphorylation as the major system for ATP production, both diuron and chlorpropham reduced ATP levels and growth. These findings indicated that both diuron and chlorpropham inhibited the photochemical production of ATP.

If photosynthetic inhibitions are responsible for reductions of growth, an external carbohydrate supply may prove protective, because the ATP required for growth could be obtained through the mitochondrial oxidative phosphorylation system. We found that when glucose was included in diuron-treated cultures, both growth and ATP levels returned to control levels, whereas inclusion of glucose in chlorpropham-treated cultures was essentially without

effect (St. John, 1971). Therefore, chlorpropham also interfered with the oxidative production of ATP, but diuron did not. Furthermore, chlorpropham had a stronger effect on ATP level than on growth for diuron, the inhibitions of growth, and ATP levels essentially paralleled each other. Thus the metabolism of diuron-treated cultures was still geared to the production of an approximately constant level of ATP, and ATP synthesis was probably not growth-limiting. Chlorpropham-treated cultures, however, did not maintain ATP in balance with growth, and ATP may have been limiting.

Our data taken collectively indicate that the luciferin-luciferase system can be adapted to studies on herbicide effects on phosphorylation processes in vivo, that effects on photophosphorylation and oxidative phosphorylation can be separated, and that herbicide effects on ATP levels can be related to growth control.

Since the physiological functions and biochemical processes required for plant growth and development are driven by energy derived from ATP, it follows that herbicides that inhibit ATP production may control growth indirectly by limiting ATP requiring biosynthetic systems. Ribonucleic acid (RNA) and protein synthesis are essential processes for plant growth and development, and, at least in *Escherichia coli*, these processes can account for up to 90 percent of the ATP expended for biosynthetic processes. When data (Moreland et al., 1969) on 14 of 22 herbicides that inhibited RNA and protein synthesis were examined, some of the strongest inhibitors of these biosynthetic reactions had been reported by various investigators to inhibit oxidative phosphorylation in isolated mitochondria. Thus, the effects of these herbicides on RNA and protein synthesis might be attributed to interference with ATP production. Gruenhagen and Moreland (1971) studied the effects of 22 herbicides on in vivo levels of ATP, orotic acid incorporation into RNA, and leucine incorporation into protein in soybean hypocotyls, using the luciferin-luciferase system to measure ATP. As expected, no herbicide was found to reduce tissue ATP levels and not inhibit RNA and protein synthesis. The correlations established between tissue ATP levels and inhibitions of RNA and protein synthesis suggested that interference with the production of energy, required to drive biosynthetic reactions, could be the mechanism through which these herbicides act.

However, herbicides could also produce phytotoxicity by interfering directly with steps in biosynthetic reaction sequences. The luciferin-luciferase enzyme system has exposed the mechanism by which such interferences could occur, and again ATP has been involved. The enzyme systems responsible for firefly light emission, amino acid activation, and fatty acid activation each form an appropriate enzyme-adenylate complex from ATP in the presence of Mg^{++} (McElroy et al., 1967). The luciferyl-adenylate complex reacts with oxygen, emitting light and releasing AMP; the amino acid-adenylate complex reacts with tRNA, forming an amino-acid-tRNA complex plus AMP: and the fatty-acid-adenylate complex reacts with CoASH, forming fatty-acid-CoA and AMP (McElroy et al., 1967).

Kinetic analyses of herbicide inhibitions of the luciferin-luciferase enzyme system in vitro done in our laboratory (unpublished data) and by Rusness and Still (1974) indicated that herbicides may be linear noncompetitive inhibitors in respect to ATP, linear competitive inhibitors in respect to D-luciferin, or linear competitive inhibitors in respect to both ATP and D-luciferin.

If the firefly luciferase system can serve as a model system to be compared to amino acid or fatty acid activation, then a second mechanism of herbicide action involving ATP can be postulated. Certain herbicides may control growth by preventing ATP use by blocking the formation of enzyme-adenylate complexes required in biosynthetic reaction sequences.

In conclusion, the luciferin-luciferase enzyme system for determination of ATP is valuable for studies on the mechanisms of herbicide action. Studies using this system have shown that certain herbicides may act by interfering with ATP production or by blocking ATP use, or possibly by both mechanisms.

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